PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY

(Chapter II of the Patent Cooperation Treaty)

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference PA0356 PCT	**FOR FURTHER ACTION**	See Form PCT/IPEA/416
International application No. PCT/GB2004/003341	International filing date *(day/month/year)* 30.07.2004	Priority date *(day/month/year)* 30.07.2003
International Patent Classification (IPC) or national classification and IPC G01N33/50, G01N33/533		
Applicant AMERSHAM BIOSCIENCES UK LIMITED et al.		

1. This report is the international preliminary examination report, established by this International Preliminary Examining Authority under Article 35 and transmitted to the applicant according to Article 36.
2. This REPORT consists of a total of 7 sheets, including this cover sheet.
3. This report is also accompanied by ANNEXES, comprising:
   a. ☒ *sent to the applicant and to the International Bureau)* a total of 11 sheets, as follows:
      ☒ sheets of the description, claims and/or drawings which have been amended and are the basis of this report and/or sheets containing rectifications authorized by this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions).
      ☐ sheets which supersede earlier sheets, but which this Authority considers contain an amendment that goes beyond the disclosure in the international application as filed, as indicated in item 4 of Box No. I and the Supplemental Box.
   b. ☐ *(sent to the International Bureau only)* a total of (indicate type and number of electronic carrier(s)) , containing a sequence listing and/or tables related thereto, in computer readable form only, as indicated in the Supplemental Box Relating to Sequence Listing (see Section 802 of the Administrative Instructions).

4. This report contains indications relating to the following items:

☒	Box No. I	Basis of the opinion
☐	Box No. II	Priority
☒	Box No. III	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
☐	Box No. IV	Lack of unity of invention
☒	Box No. V	Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
☒	Box No. VI	Certain documents cited
☐	Box No. VII	Certain defects in the international application
☐	Box No. VIII	Certain observations on the international application

Date of submission of the demand	Date of completion of this report
11.02.2005	28.11.2005
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized Officer Pinheiro Vieira, E Telephone No. +49 89 2399-7865

INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY

International application No.
PCT/GB2004/003341

Box No. I Basis of the report

1. With regard to the **language**, this report is based on the international application in the language in which it was filed, unless otherwise indicated under this item.

   ☐ This report is based on translations from the original language into the following language , which is the language of a translation furnished for the purposes of:
   - ☐ international search (under Rules 12.3 and 23.1(b))
   - ☐ publication of the international application (under Rule 12.4)
   - ☐ international preliminary examination (under Rules 55.2 and/or 55.3)

2. With regard to the **elements*** of the international application, this report is based on *(replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report)*:

Description, Pages

1-4, 6-11, 13-15, 17-28	as originally filed
5, 12, 16	received on 04.08.2005 with letter of 03.08.2005

Claims, Numbers

1-26	received on 04.08.2005 with letter of 03.08.2005

Drawings, Sheets

1/5-5/5	as originally filed

☐ a sequence listing and/or any related table(s) - see Supplemental Box Relating to Sequence Listing

3. ☒ The amendments have resulted in the cancellation of:
   - ☐ the description, pages
   - ☒ the claims, Nos. 27
   - ☐ the drawings, sheets/figs
   - ☐ the sequence listing *(specify)*:
   - ☐ any table(s) related to sequence listing *(specify)*:

4. ☐ This report has been established as if (some of) the amendments annexed to this report and listed below had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).
   - ☐ the description, pages
   - ☐ the claims, Nos.
   - ☐ the drawings, sheets/figs
   - ☐ the sequence listing *(specify)*:
   - ☐ any table(s) related to sequence listing *(specify)*:

* *If item 4 applies, some or all of these sheets may be marked "superseded."*

Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

☐ the entire international application,

☒ claims Nos. 15-24

because:

☒ the said international application, or the said claims Nos. 15-24 relate to the following subject matter which does not require an international preliminary examination (specify):

see separate sheet

☐ the description, claims or drawings *(indicate particular elements below)* or said claims Nos. are so unclear that no meaningful opinion could be formed *(specify)*:

☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

☐ no international search report has been established for the said claims Nos.

☐ the nucleotide and/or amino acid sequence listing does not comply with the standard provided for in Annex C of the Administrative Instructions in that:

the written form	☐	has not been furnished
	☐	does not comply with the standard
the computer readable form	☐	has not been furnished
	☐	does not comply with the standard

☐ the tables related to the nucleotide and/or amino acid sequence listing, if in computer readable form only, do not comply with the technical requirements provided for in Annex C-*bis* of the Administrative Instructions.

☐ See separate sheet for further details

Box No. V Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	2,3,7-13
	No:	Claims	1,4-6,14-26
Inventive step (IS)	Yes:	Claims	13
	No:	Claims	1-12,14-26
Industrial applicability (IA)	Yes:	Claims	1-14,25,26
	No:	Claims	15-24

2. Citations and explanations (Rule 70.7):

see separate sheet

Box No. VI Certain documents cited

1. Certain published documents (Rule 70.10)

and / or

2. Non-written disclosures (Rule 70.9)

see separate sheet

Re Item III.

Claims 16-25 relate to subject-matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of these claims (Article 34(4)(a)(I) PCT).

Re Item V.

V.1 Reference is made to the following documents:

D1 : WO 03/020294 A (SCHMIDT, ALFRED; WIELAND, HEINRICH) 13 March 2003 (2003-03-13)

D2 : STRESSER D M ET AL: "A HIGH-THROUGHPUT SCREEN TO IDENTIFY INHIBITORS OF AROMATASE (CYP19)" ANALYTICAL BIOCHEMISTRY, ACADEMIC PRESS, SAN DIEGO, CA, US, vol. 284, no. 2, 2000, pages 427-430, XP000979096 ISSN: 0003-2697

D3: WO 02/099424 A (AMERSHAM BIOSCIENCES UK LIMITED; SMITH, JOHN, ANTHONY; WEST, RICHARD,) 12 December 2002 (2002-12-12)

D1 discloses compounds having at least one detectable group where the compounds comprise an aromatase substrate. It further relates to diagnostic and therapeutic methods using said compounds.

D2 concerns a fluorometric substrate for aromatase (O-benzylfluorescein benzyl ester) and it's use in a screening method.

D3 discloses acridone derivatives and their use as fluorescent labels in methods of screening and methods for labelling substrates.

V.2 Novelty, inventive step and industrial applicability (Art. 33 PCT).

2.1 The present application concerns compounds containing a fluorescent dye molecule (**R**) coupled to a substrate for aromatase (**S**) via a linker (**L**) with the formula **R-L-S** characterized in that the fluorescence signal of the compounds changes in respect of fluorescence lifetime when the compound is acted upon by an enzyme with

aromatase activity; use of compound for measuring aromatase activity in sample; diagnostic and screening methods.

2.2 The present application does not meet the criteria of Article 33(1) PCT, because the subject-matter of claims 1, 4-6 and 14-26 is not new in the sense of Article 33(2) PCT in view of D2. Document D2 disclose changes of the fluorescent signal in respect of fluorescence lifetime when the compound is acted upon by an enzyme with aromatase activity.

2.3 It is at present not apparent to which problem the compounds of **formula (I)** where **R** is an acridone dye or quinacridone (claims 2 and 3), where **L** is the linker as defined in claims 7 or 8, and where **S** is the substrate as given in claims 9-12, could be a solution. Acridone dye derivatives are known from D3 as suitable fluorescence labels for labelling and lifetime detection of a target material, the substrates defined by claims 9-12 are known from D2, and the linkers **L** as claimed in claims 7 or 8 are well known chemical linkers for the skilled man.
The subject matter of claims 2, 3 and 7-12 is therefore, not inventive.

2.4 The problem to be solved by claim 13 in view of the closest prior art document D1, can be seen as the provision of further labelled substrates for aromatase. The Applicant solves the problem by providing the compounds of **formula XX** where the label is an acridone dye molecule linked via a linker to testosterone.

The application differs from D1 in that the label is an acridone dye. All over D1 description's there are references to labelled aromatase substrates (also testosterone) detectable by spectroscopic methods, and imaging methods adapted to the detectable group. Nevertheless, this document is silent as to the use of acridone dyes conjugated to testosterone.
Although, document D3 discloses acridone florescent dyes and their use as fluorescent probes this document does not suggest acridone dyes bound to the particular substrate testosterone.
D2 concerns another substrate labelled with flourescein.
In view of the prior art documents D1-D3 it is not obvious to provide the compounds of **formula XX** where testosterone is labelled with an acridone dye. Therefore, claim

14 is inventive.

2.5 The subject matter of claims 1-14, 25 and 26 is industrial applicable.

Claims

1. A compound of Formula 1:

R-L-S

(I)

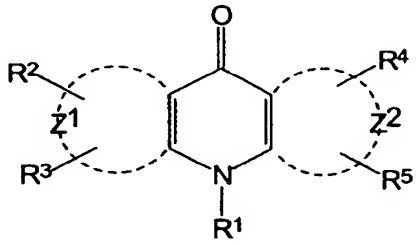
wherein R is a fluorescent dye molecule;

L is an optional linkage group containing one or more atoms comprising hydrocarbon chains which may also contain other atoms such as N, O and S; and

S is molecule comprising a substrate group of the enzyme aromatase

characterised in that the fluorescence signal of said compound changes in respect of fluorescence lifetime when the compound is acted upon by an enzyme with aromatase activity.

2. A compound according to claim 1 wherein said R is an acridone dye of Formula II:



(II)

wherein:

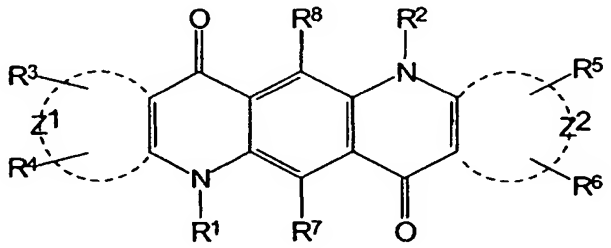
groups R^2 and R^3 are attached to the Z^1 ring structure and groups R^4 and R^5 are attached to the Z^2 ring structure;

Z^1 and Z^2 independently represent the atoms necessary to complete one or two fused ring aromatic or heteroaromatic systems, each ring having five or six atoms

selected from carbon atoms and optionally no more than two atoms selected from oxygen, nitrogen and sulphur;

R^1, R^2, R^3, R^4 and R^5 are independently selected from hydrogen, halogen, amide, hydroxyl, cyano, amino, mono- or di-C_1-C_4 alkyl-substituted amino, sulphydryl, carbonyl, C_1-C_6 alkoxy, aryl, heteroaryl, C_1-C_{20} alkyl, aralkyl; the group -E-F where E is a spacer group having a chain from 1-60 atoms selected from the group consisting of carbon, nitrogen, oxygen, sulphur and phosphorus atoms and F is a target bonding group; and the group -$(CH_2$-$)_n$Y where Y is selected from sulphonate, sulphate, phosphonate, phosphate, quaternary ammonium and carboxyl and n is zero or an integer from 1 to 6.

3. A compound according to claim 1 wherein R is a quinacridone dye of Formula III:



(III)

wherein:

groups R^3 and R^4 are attached to the Z^1 ring structure and groups R^5 and R^6 are attached to the Z^2 ring structure;

Z^1 and Z^2 independently represent the atoms necessary to complete one or two fused ring aromatic or heteroaromatic systems, each ring having five or six atoms selected from carbon atoms and optionally no more than two atoms selected from oxygen, nitrogen and sulphur;

R^1, R^2, R^3, R^4, R^5, R^6, R^7 and R^8 are independently selected from hydrogen, halogen, amide, hydroxyl, cyano, amino, mono- or di-C_1-C_4 alkyl-substituted amino, sulphydryl, carbonyl, carboxyl, C_1-C_6 alkoxy, aryl, heteroaryl, C_1-C_{20} alkyl, aralkyl; the group -E-F where E is a spacer group having a chain from 1-60

atoms selected from the group consisting of carbon, nitrogen, oxygen, sulphur and phosphorus atoms and F is a target bonding group; and the group $-(CH_2-)_nY$ where Y is selected from sulphonate, sulphate, phosphonate, phosphate, quaternary ammonium and carboxyl and n is zero or an integer from 1 to 6.

4. A compound according to any of claims 1 to 3 wherein L is a linker group containing from 1 to 40 linked atoms selected from carbon atoms which may optionally include one or more groups selected from –NR'-, -O-, -S-, -CH=CH-, -C≡C-, -CONH- and phenylenyl groups, wherein R' is selected from hydrogen and C1 to C4 alkyl.

5. A compound according to any of claims 1 to 4, wherein L is a linker group containing from 2 to 30 atoms.

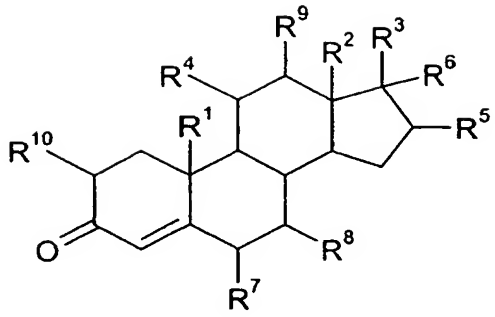
6. A compound according to any of claim 1 to 5, wherein L is a linker group containing from 6 to 20 atoms.

7. A compound according to any of claims 1 to 6, wherein L is a linker group selected from the group: $\{(-CHR'-)_p-Q-(-CHR'-)_r\}_s$

where each Q is selected from CHR', NR', O, -CH=CH-, Ar and –CONH-;
each R' is independently hydrogen or C_1 to C_4 alkyl;
each p is independently 0 to 5;
each r is independently 0 to 5;
and s is either 1 or 2.

8. A compound according to claim 7, wherein Q is selected from the group consisting of -CHR'-, -O- and –CONH-, where R' is hydrogen or C_1 to C_4 alkyl.

9. A compound according to any preceding claim wherein S is a substrate group of the enzyme aromatase of formula IX



(IX)

wherein:

R^1 and R^2 are selected from H and methyl;

R^3 is selected from H, C_1-C_8 alkyl, cyano, $-(CH_2)_k-OR^a$; $-(CH_2)_k-COOR^a$; $-(CH_2)_k-SO_3R^a$; $-(CH_2)_k-CHO$, $-(CH_2)_k-NR^bR^c$ and $-(CH_2)_k-COR^d$;

R^4 is selected from H, $-COR^a$ and hydroxyl;

R^5 is selected from H, $-COR^a$, hydroxyl, cyano and halide;

R^6 is selected from H and hydroxyl;

R^7, R^8 and R^9 are independently selected from H, $-COR^a$ and hydroxyl;

R^{10} is selected from H and halide; and

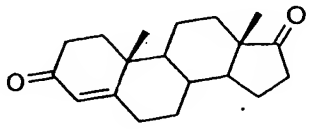
where R^a is selected from H and C1 – C4 alkyl, optionally substituted with OH; R^b and R^c are selected from H and C_1-C_4 alkyl;

R^d is selected from C_1-C_8 alkyl or C_1-C_8 alkyl optionally substituted with $COOR^a$, OH, OR^a or SO_3R^a;

and k is zero or an integer from 1 to 8.

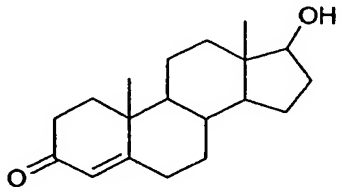
10. A compound according to claim 9 wherein Group S is a steroid selected from the group of steroid families consisting of 4-androsten-3-one, 4-cholesten-3-one, 4-estren-3-one and 4-pregnen-3-one derivatives.

11. A compound according to any of claims 1 to 10 wherein S is androstenedione of Formula X or a derivative thereof.



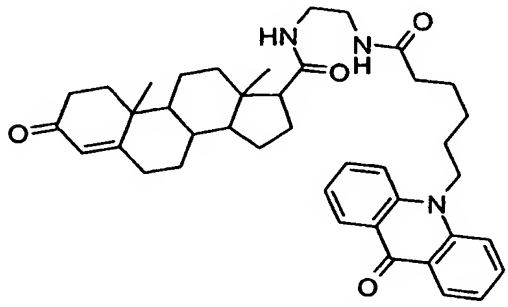
(X)

12. A compound according to any of claims 1 to 10 wherein S is testosterone of Formula XI or a derivative thereof.



(XI)

13. A compound according to any preceding claim of Formula XX



(XX)

14. A method for measuring aromatase activity in a sample, the method comprising the steps of:

i) measuring the fluorescence lifetime of a compound according to any preceding claim prior to adding it to said sample;

ii) adding said compound to said sample under conditions which favour aromatase activity, and

iii) measuring a change in fluorescence lifetime of said compound following step ii);

wherein said change in fluorescence lifetime can be used to determine aromatase activity.

15. A method according to claim 14 wherein the sample is selected from the group consisting of extract, cell, tissue and organism.

16. A method of screening for a test agent whose effect upon the activity of aromatase is to be determined, said method comprising the steps of:

i) performing the method of claim 14 or 15 in the presence of said agent; and

ii) comparing the activity of said aromatase in the presence of the agent with a known value for the activity of aromatase in the absence of the agent;

wherein a difference between the activity of the aromatase in the presence of the agent and said known value in the absence of the agent is indicative of the effect of the test agent upon the activity of aromatase.

17. The method according to claim 16, wherein the known value is stored upon an electronic database.

18. A method of screening for a test agent whose effect upon the activity of aromatase is to be determined, said method comprising the steps of:

i) performing the method of claim 16 or 17 in the presence and in the absence of the agent; and

ii) determining the activity of said enzyme in the presence and in the absence of the agent;

wherein a difference between the activity of aromatase in the presence and in the absence of the agent is indicative of the effect of the test agent upon the activity of aromatase.

19. The method according to claim 17 wherein said difference in activity between the activity of aromatase in the absence and in the presence of the agent is normalised, stored electronically and compared with a value of a reference compound.

20. A method for measuring the distribution of a compound of any of claims 1 to 13 within a tissue, wherein the compound is capable of being taken up by a living cell within said tissue, the method comprising the steps of:

i) measuring the fluorescence lifetime of the compound in a cell-free environment or a parental host cell;

ii) adding the compound to one or more cells or a cell engineered to over-express aromatase, and

iii) measuring the fluorescence lifetime of the compound following step ii);

wherein a change in fluorescence lifetime indicates aromatase activity and can be used to determine the distribution of the compound.

21. A method according to claim 20, wherein the distribution of the compound within the tissue of a first subject is compared with the distribution of the compound within the tissue of a second subject.

22. The method of claim 21, wherein said subject is selected from the group consisting of mammal, plant, insect, fish, bird, fly, nematode and algae.

23. The method of claim 22, wherein the mammal is a mouse or a rat.

24. Use of a compound according to any of claims 1 to 13 for measuring aromatase activity as an *in vitro* or an *in vivo* imaging probe.

25. A method of diagnosing a disease caused by an increase in aromatase activity in a subject using the method according to claim 14, comprising comparing the activity of aromatase in a sample taken from a first subject with the activity in a sample taken from a second healthy control subject, wherein any increase in activity measured in the sample taken from the first subject relative to the second healthy control subject is indicative of disease.

26. Kit comprising:

i) a compound according to any of claims 1 to 13;

ii) an assay buffer; and optionally

iii) a stop buffer.

detection for distinguishing labelled compounds. Preferably the improved assays display more than one of these features and preferably they display all of these features. The present invention seeks to provide novel reagents and methods for performing such an assay.

Summary of Invention

According to a first aspect of the present invention, there is provided a compound of Formula I:

R-L-S

(I)

wherein R is a fluorescent dye molecule;

L is an optional linkage group containing one or more atoms comprising hydrocarbon chains which may also contain other atoms such as N, O and S; and

S is a molecule comprising a substrate group of the enzyme aromatase

characterised in that the fluorescence signal of said compound changes in respect of fluorescence lifetime when the compound is acted upon by an enzyme with aromatase activity.

A range of fluorescent labels are commercially available which could be used as a fluorescent reporter moiety R in accordance with the present invention. Examples include, but are not limited to, oxazine (e.g. MR 121, JA 242, JA 243) and rhodamine derivatives (e.g. JA 165, JA 167, JA 169) as described in WO 02/081509. Other examples (as described in WO 02/056670) include, but are not limited to Cy5, Cy5.5 and Cy7 (Amersham); merocyanine (Few Chemicals), IRD41 and IRD700 (Licor); NIR-1 and IC5-OSu (Dojindo); Alexa fluor 660 & Alexa fluor 680 (Molecular Probes); LaJolla Blue (Diatron); FAR-Blue, FAR-Green One & FAR-Green Two (Innosense); ADS 790-NS and ADS

Suitably, conventional detection methods can be employed to measure fluorescence intensity and/or the lifetime of the label. These methods include instruments using photo-multiplier tubes as detection devices. Several approaches are possible using these methods; e.g.

i) methods based upon time correlated single photon counting (cf. Principles of Fluorescence Spectroscopy, (Chapter4) ed. J R Lakowicz, Second Edition, 1999, Kluwer/Academic Press)

ii) methods based upon frequency domain/phase modulation (cf. Principles of Fluorescence Spectroscopy, (Chapter5) ed. J R Lakowicz, Second Edition, 1999, Kluwer/Academic Press)

iii) methods based upon time gating (cf. Sanders et al., (1995) Analytical Biochemistry, 227 (2), 302-308).

Measurement of fluorescent intensity may be performed by means of a charge coupled device (CCD) imager, such as a scanning imager or an area imager, to image all of the wells of a multiwell plate. The LEADseeker™ (Amersham Biosciences, UK) system features a CCD camera allowing imaging of high density microtitre plates in a single pass. Imaging is quantitative and rapid, and instrumentation suitable for imaging applications can now simultaneously image the whole of a multiwell plate.

According to a fifth aspect of the present invention, there is provided a method for measuring the distribution of a compound as hereinbefore described within a tissue, wherein the compound is capable of being taken up by a living cell within the tissue, the method comprising the steps of:

i) measuring the fluorescence lifetime of the compound in a cell-free environment or a parental host cell;

ii) adding the compound to one or more cells or a cell engineered to over-express aromatase, and

iii) measuring the fluorescence lifetime of the compound following step ii);

wherein a change in fluorescence lifetime indicates aromatase activity and can be used to determine the distribution of the